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The Greenland shark: a new challenge for the oxidative stress theory of ageing?

David Costantini[a,b,c*], Shona Smith[b], Shaun S. Killen[b], Julius Nielsen[d], John F. Steffensen[d]

[a] Department of Biology, University of Antwerp, Universiteitsplein 1, 2610 Wilrijk, Belgium

[b] Institute for Biodiversity, Animal Health and Comparative Medicine, University of Glasgow, Glasgow G12 8QQ, UK

[c] Current Address: Department of Evolutionary Ecology, Leibniz Institute for Zoo and Wildlife Research, Alfred-Kowalke-Str. 17, 10315 Berlin, Germany

[d] Marine Biological Section, Department of Biology, University of Copenhagen, Strandpromenaden 5, DK-3000 Helsingør, Denmark

* email: davidcostantini@libero.it

ABSTRACT

The free radical theory of ageing predicts that long-lived species should be more resistant to oxidative damage than short-lived species. Although many studies support this theory, recent studies found notable exceptions that challenge the generality of this theory. In this study, we have analysed the oxidative status of the Greenland shark (*Somniosus microcephalus*), which has recently been found as the longest living vertebrate animal known to science with a lifespan of at least 272 years. As compared to other species, the Greenland shark had body mass-corrected values of muscle glutathione peroxidase and red blood cells protein carbonyls (metric of protein oxidative damage) above 75 percentile and below 25 percentile, respectively. None of the biochemical metrics of oxidative status measured in either skeletal muscle or red blood cells were correlated with maximum lifespan of species. We propose that the values of metrics of oxidative status we measured might be linked to ecological features (e.g., adaptation to cold waters and deep dives) of this shark species rather to its lifespan.

Keywords: ageing, lifespan, oxidative stress, sharks, vertebrates

1. Introduction

Some species live for hundreds of years; the lifetime of some other species is limited to only a few hours. This surprising variation in longevity has intrigued biologists for decades. A key issue that has generated an overwhelming body of research is the underlying cause of senescence and of this dramatic variation in maximum lifespan. In 1956, Denham Harman suggested that the production of free radicals might be responsible for cell senescence because free radicals can damage vital biomolecules like proteins, lipids and nucleic acids, causing cell senescence. The free radical theory of ageing has since been refined and a number of iterations have been presented, including the mitochondrial theory of ageing (Harman, 1972) and the oxidative stress hypothesis of ageing (Yu and Yang, 1996).

One commonly used method of unraveling the factors linked to species longevity is to compare a given biochemical metric of oxidative status across species exhibiting variation in maximum lifespan. In this context, many studies concluded that long-lived species are more resistant to oxidative damage (e.g., cell composition with biomolecules less sensitive to oxidation) than short-lived species and that long-lived species have lower antioxidant levels than short-lived species because they produce less free radicals (Hulbert et al., 2007; Pamplona and Costantini, 2011). As with all apparently general patterns in biology, there are important exceptions that challenge the generality of the theory. Contrary to the predictions of the free radical theory of ageing, naked mole rats (*Heterocephalus glaber*), a long-living rodent, do not have exceptionally high levels of antioxidant protection (Andziak et al., 2005) and have elevated damage to DNA, lipids and proteins (Andziak et al., 2006) as compared to similar-sized mice. Another example is the olm (*Proteus anguinus*), a small cave

salamander that has evolved an extreme life history strategy with a predicted maximum lifespan of over 100 years. Surprisingly, neither its basal metabolism nor oxidative damage and activities of antioxidant enzymes explain why this species sits as an outlier in the relationship between amphibian size and longevity (Issartel et al., 2009; Voituron et al., 2011). These and other experimental findings led some authors to challenge the oxidative stress theory of ageing (Speakman and Selman, 2011).

The Greenland shark (*Somniosus microcephalus*) is one of the world's largest predatory sharks (maximum body length > 5 meters), mainly distributed in Arctic and subarctic regions of the North Atlantic (Bigelow and Schroeder, 1948; Nielsen et al., 2014). Surprisingly little is known about the general biology of Greenland shark (Hansen 1952, 1963), but a recent study based on radiocarbon dating of the sharks eye lens found lifespan of at least 272 years and that it has a very long phase before sexual maturity (around 130 years until maturity for females, Nielsen et al., 2016), which makes the Greenland shark the longest living vertebrate animal known to science. The Greenland shark is clearly suitable for evaluating whether its oxidative status explains its lifespan or is rather a consequence of its ecology (e.g., exposure to repeated episodes of hypoxia associated with deep water dives). We have therefore measured two metrics of oxidative status in both blood and muscle of Greenland sharks and compared their levels to those of species with different maximum lifespans.

2. Material and methods

2.1. Tissue collection from sharks

Samples from non-sexually mature Greenland sharks (n = 11 individuals, 2 males and 9 females; Table 1) were collected in 2012 in Ammassalik Fjord, south-eastern Greenland

from the Danish research vessel Dana. Sharks were caught on bottom long lines at depths between 300 and 500 m. Immediately after capture, a sample of blood (ca. 100 ml) was collected, centrifuged for 3 minutes at 10,000 rpm and stored at -18°C while on the field. Samples of skeletal muscle (white muscle) were collected from the dorsal side above the gills and stored at -18°C while on the field. All samples were then stored at -80°C. Body mass of each individual shark was measured on a digital scale except for four sharks. For these sharks body mass was calculated according to Nielsen et al. (2014). Animals were either released with tags or euthanized for research purposes. Sampling of sharks was carried out in accordance with laws and regulations and with authorization from the Government of Greenland (Ministry of Fisheries, Hunting & Agriculture, document number 935119).

2.2. Laboratory analyses

Samples of skeletal muscle were homogenised in ice cold PBS (supplemented with 20% (v/v) glycerol and 0.2 mM phenylmethylsulfonyl fluoride as an inhibitor of proteases) using a pestle. Samples were then sonicated for 10 minutes and then centrifuged for 10 minutes at 10,000 rpm. The supernatant was split into different aliquots, which were stored at -80 °C for later analyses of protein carbonyls (metric of protein oxidative damage) and activity of glutathione peroxidase, two metrics that were found to be linked to species lifespan (Pamplona and Costantini, 2011; Halliwell and Gutteridge, 2015). Protein carbonyls (PCs) were measured according to Levine et al. (1990). Carbonyls (C=O) are introduced into proteins from free radicals or via reactions with lipid peroxidation products (malondialdehyde and hydroxynonenal) or carbohydrates; protein carbonylation is mostly irreversible (Halliwell and Gutteridge, 2015). Nucleic

110 acids were removed by adding 1 volume of a 10% solution of streptomycin sulfonate to
111 9 volumes of sample. PCs were then derivatised to 2,4-dinitrophenylhydrazone by
112 reaction with 2,4-dinitro-phenylhydrazine (DNPH). The Ransel assay (RANDOX
113 Laboratories, Crumlin, UK) was used to quantify the activity of glutathione peroxidase
114 (GPX). Glutathione peroxidase uses the reduced form of glutathione to reduce
115 peroxides and hydroperoxides to water and alcohols, respectively (Halliwell and
116 Gutteridge, 2007). The Ransel assay is based on the method of Paglia and Valentine
117 (1967) using cumene hydroperoxide as a substrate. Measures of protein carbonyls and
118 GPX were standardised by expressing the concentrations per mg of proteins as
119 measured by the Bradford protein assay (Bio-Rad Laboratories, Hercules, USA) using a
120 standard curve of bovine serum albumin. Hence, protein carbonyls were expressed as
121 nmol/mg proteins, while GPX was expressed as Units/mg proteins.
122
123 *2.3. Data collection from literature*
124 A literature search for studies in which protein carbonyls and glutathione peroxidase
125 (GPX) were analysed using our same protocols, respectively, was conducted on both
126 Web of Science and Scopus using a combination of keywords ("protein carbonyls",
127 "GPX", "muscle", "red blood cells"). Data are reported in Table 2.
128 Data on body mass and maximum lifespan were collected from
129 genomics.senescence.info/species/ or fishbase.org/ when not reported in the articles
130 selected for this study. Longevity of *Hypophtalmichthys molitrix* and of *Hoplias*
131 *malabaricus* were obtained, respectively, from
132 animaldiversity.ummz.umich.edu/accounts/Hypophthalmichthys_molitrix/ and Novaes
133 and Carvalho (2011).

134

135 *2.4. Statistical analyses*

136 All statistical analyses were performed using SPSS version 22 (Chicago, USA). Values
137 of biochemical metrics, body mass and maximum lifespan (Table 2) were transformed
138 as log (x) or log (x + 1) before analyses in order to improve normality of distribution.
139 Similar results were obtained including untransformed variables in all models (data not
140 shown). To describe the distribution of PCs and GPX across species while controlling
141 for multiple sources of variation, we extracted residuals of each biochemical measure
142 (included in the models as dependent variables) from Linear Mixed Models (LMMs)
143 including 'study' as a random factor (because some articles analysed multiple species
144 for muscle PCs and GPX and for red blood cell GPX) and two independent fixed
145 factors: 1) origin of the animals (i.e. wild or cultured; humans were included in the
146 group of artificially selected strains); and 2) taxonomic class (fish, amphibians, reptiles,
147 birds or mammals). Mean adult body mass of each species was included as a covariate
148 to control for any allometric effects. Given that body mass data were obtained from
149 different sources, we opted to run additional LMMs where body mass was not included
150 in the models in order to control for any bias. In order to assess whether species that live
151 longer have lower production of PCs and lower levels of GPX in both skeletal muscle
152 and red blood cells, we calculated correlations between residuals of biochemical metrics
153 and residuals of maximum lifespan obtained from a linear regression of log-transformed
154 maximum lifespan onto log-transformed body mass. Given the moderate sample size,
155 we present results from Spearman test. Note that results were similar where Pearson test
156 was used. In preliminary analyses, we also used LMMs to test the covariation between a
157 given biochemical metric and maximum lifespan while controlling for potential sources

of variation, like taxonomic class or body mass. Given that results were similar to those of bivariate correlations (data not shown), we opted to present only data from correlations for easy of presentation.

3. Results

Compared to other species included in this analysis, the Greenland shark has particularly high values of PCs and GPX in muscle (both above 75 percentile; Table 3). When among species differences in body mass are corrected for, compared to other species, the Greenland shark has high muscle GPX (above 75 percentile) and low values of red blood cell PCs (below 25 percentile; Table 3). In contrast, values of muscle PCs now sit in the middle of the whole variation of our sample. Activity of GPX in red blood cells sits in the middle of the whole variation of our sample irrespective of whether body mass is controlled for or is not.

None of the biochemical metrics of oxidative status were related to species maximum lifespan (Spearman correlation coefficients: range -0.18 to 0.18, $p \geq 0.30$), regardless of whether body mass was or was not included in the models (Fig. 1).

4. Discussion

Results of our comparative study indicated that the oxidative status of Greenland shark appears to differ from that of other species. This is particularly evident from body mass-corrected values of muscle GPX and red blood cells PCs, which were above 75 percentile and below 25 percentile, respectively. These characteristics of the Greenland shark do not seem, however, to be the reason for its long lifespan as compared to other species. In fact, none of the metrics of oxidative status in either skeletal muscle or red

182 blood cells were correlated with species maximum lifespan. A question then is why
183 Greenland sharks have high muscle GPX and low red blood cell PCs. The Greenland
184 shark inhabits cold waters, mostly in the range -1.8 to 10°C (Stokesbury et al., 2005).
185 Moreover, data from 25 years of surveys show that Greenland sharks are usually caught
186 at 400–700 m, but were found at all depths between 100 and 1,200 m (Nielsen et al.,
187 2014). Hence, the oxidative status of the Greenland shark might have been shaped to
188 some degree by adaptation to a life in the cold Arctic waters and to deep dives. For
189 example, many species that are faced with repeated episodes of hypoxia/reoxygenation
190 (as typical of diving animals) tend to have high basal levels of antioxidant defences in
191 order to protect them against high free radical production that occurs during
192 reoxygenation (Costantini, 2014). This might be one reason that explains why
193 Greenland sharks have high GPX (an antioxidant enzyme) as compared to the other
194 species included in this study.

195 Estimates of maximum lifespan include both the pre- and post-reproductive
196 phases. Hence, maximum lifespan of long-lived species may be inflated with the
197 addition of a period of life over which individuals are under a waning force of selection
198 (Reznick et al., 2006). Conversely, evolution would have shaped antioxidant
199 mechanisms to impact on the organism only in the period of life where selection is
200 stronger. This is particularly relevant for the Greenland shark. It has in fact a very long
201 phase before sexual maturity (Nielsen et al., 2016) during which selection would be
202 expected to be particularly long and sustained. Hence, individuals poorly protected
203 against oxidative stress might be rapidly eliminated by natural selection. All Greenland
204 shark individuals included in this study were not sexually mature. However, growth
205 does not appear to be a reason for the high GPX because sharks have an indeterminate

growth, i.e., they keep growing all their life long. Also many studies found juveniles to have lower GPX than adults (Costantini, 2014). Further studies will clearly be needed to unravel the reasons for the high activity of GPX in skeletal muscle and the low level of protein damage in red blood cells. Although both protein carbonyls and GPX were previously found to be linked to species lifespan (Pamplona and Costantini, 2011; Halliwell and Gutteridge, 2015), it will be important to collect data on other metrics of oxidative status (e.g., DNA damage and repair) that might reveal routes through which Greenland sharks pave the way to their exceptional longevity. Species-based approaches may, however, hide within-species variation in the association between oxidative status and longevity that has been found in several vertebrate species (Costantini, 2014). Thus, a proper understanding of the oxidative stress theory of ageing will require much further study, in particular a combination of comparative species- and individual-based approaches tailored to elucidate the association between oxidative status and longevity, and whether the nature of the association is species-specific rather than general.

In conclusion, our study showed that levels of GPX and oxidative damage to proteins (protein carbonyls) in muscle and red blood cells were not associated with species lifespan, suggesting that these two metrics of oxidative status do not explain the exceptional longevity of the Greenland shark. The high and low values of muscle GPX and red blood cells PCs, respectively, we found in the Greenland shark as compared to other vertebrate species might be explained by other ecological features of this shark species, such as the adaptation to cold waters and deep dives.

Competing interests

The authors declare no competing financial interests.

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390

391 Table 1. Descriptive statistics of Greenland sharks included in our study. X = the
392 biological matrix collected from a single specimen. RBCs = red blood cells; PCs =
393 protein carbonyls (expressed as nmol/mg proteins); GPX = glutathione peroxidase
394 (expressed as Units/mg proteins). * For these sharks body mass was calculated
395 according to Nielsen et al. (2014). Mean total length (TL) was 327 cm (SD = 38; n =
396 11) and mean body mass (BM) was 343 kg (SD = 123; n = 11).

397

398

Sex	**TL (cm)**	**BM (kg)**	**RBC PCs**	**Muscle PCs**	**RBC GPX**	**Muscle GPX**	**RBCs**	**Muscle**
F	346	416	7.0		0.008		×	
F	306	246	3.1	10.5	0.007	0.091	×	×
F	264	168	1.9	4.5	0.006	0.099	×	×
F	386	560	7.0	3.1	0.004	0.118	×	×
F	365	430		1.8		0.059		×
F	336	338	3.3	3.4	0.007	0.070	×	×
F	355	435*	6.9		0.013		×	
F	351	452	2.8	6.3	0.011	0.075	×	×
F	302	262*	7.8		0.007		×	
M	290	231*	3.3	9.2	0.006	0.142	×	×
M	291	234*	3.6		0.006		×	

399

400

401 Table 2. Summary information of species and articles included in the comparative
402 work. MLS = maximum lifespan; rbcs = red blood cells; PCs = protein carbonyls
403 (expressed as nmol/mg proteins); GPX = glutathione peroxidase (expressed as Units/mg
404 proteins).

Class	Scientific name	Origin	Body mass (g)	MLS (years)	PCs muscle	PCs rbcs	GPX muscle	GPX rbcs	Article
Amphibians	Pelophylax ridibundus	Wild	39.5	11			0.0186		Feidantsis et al., 2013
Amphibians	Proteus anguinus	Captivity	10.6	102			0.0041		Issartel et al., 2009
Amphibians	Calotriton asper	Captivity	4.3	26			0.0041		Issartel et al., 2009
Birds	Taeniopygia guttata	Captivity	15.1	12		7.594			Costantini, unpublished data
Birds	Bubo bubo	Wild	2686	68				0.6110	Espín Luján, 2013
Birds	Gyps fulvus	Wild	7436	41.4				0.4490	Espín Luján, 2013
Birds	Coturnix japonica	Captivity	235.4	6		9.608		0.0072	Marasco et al., 2013
Birds	Fulica atra	Wild	734	20.6				0.7240	Martinez-Haro et al., 2011
Birds	Aythya ferina	Wild	823	23.2				0.2640	Martinez-Haro et al., 2011
Birds	Anas platyrhynchos	Wild	1048	29.1				0.3080	Martinez-Haro et al., 2011
Birds	Gallinula chloropus	Wild	392	18.6				0.5800	Martinez-Haro et al., 2011
Birds	Columba livia	Captivity	417	35	0.380				Montgomery et al., 2011
Birds	Melopsittacus undulatus	Captivity	26	21	0.295				Montgomery et al., 2012
Birds	Nymphicus hollandicus	Captivity	83.2	35	0.823				Montgomery et al., 2012
Birds	Coturnix japonica	Captivity	235.4	6	0.884				Montgomery et al., 2012
Birds	Coturnix chinensis	Captivity	45.4	5	0.819				Montgomery et al., 2012
Birds	Agapornis sp.	Captivity	48.9	16.1	1.528				Montgomery et al., 2012
Birds	Aptenodytes patagonicus	Wild	14000	26			0.0081		Rey et al., 2008
Birds	Cyanistes caeruleus	Wild	11.08	14.6		3.824		0.1257	Smith, 2016
Fish	Scophthalmus maximus	Captivity	113.3	26	6.288				Abele et al., 2007
Fish	Cyprinus carpio	Captivity	10600	47		11.030			García-Medina et al., 2013
Fish	Morone saxatilis	Captivity	31350	30			0.0105		Grim et al., 2013
Fish	Carassius auratus	Captivity	52.5	41	3.370				Kubrak et al., 2012
Fish	Hoplias malabaricus	Wild	246.32	9.3	3.000				Monteiro et al., 2013
Fish	Oncorhynchus mykiss	Captivity	663	11	0.260				Passi et al., 2004

Fish	Dicentrarchus labrax	Wild	1217	15	0.240				Passi et al., 2004
Fish	Somniosus microcephalus	Wild	343000	272	5.540	4.675	0.0936	0.0074	Present study
Fish	Barbus barbus	Captivity	12000	15			0.0190		Radi et al., 1985
Fish	Cyprinus carpio	Captivity	10600	47			0.0050		Radi et al., 1985
Fish	Carassius carassius	Captivity	3000	10			0.0700		Radi et al., 1985
Fish	Ctenopharyngodon idella	Captivity	1450	21			0.0019		Radi et al., 1985
Fish	Hypophtalmichthys molitrix	Captivity	450	20			0.0250		Radi et al., 1985
Fish	Sparus aurata	Captivity	9460	11			0.0023		Sanz et al., 2012
Mammals	Mus musculus	Captivity	20.5	4	0.995				Kaczor et al., 2007
Mammals	Lepus europaeus	Wild	4175	10.7			0.2100		Linsak et al., 2013
Mammals	Homo sapiens	Captivity	62000	122.5		4.670			Luqman and Rizvi, 2006
Mammals	Mus musculus	Captivity	17.6	4		3.150			Maity et al., 2013
Mammals	Rattus norvegicus	Captivity	343	3.8	0.696				Montgomery et al., 2011
Mammals	Homo sapiens	Captivity	62000	122.5	3.444				Pansarasa et al., 1999
Mammals	Rattus norvegicus	Captivity	240	3.8		1.100			Qujeq et al., 2005
Mammals	Equus caballus	Captivity	421000	57				0.0469	Williams et al., 2004
Mammals	Spermophilus tridecemlineatus	Captivity	230	7.9	0.567				Woods and Storey, 2005
Reptiles	Lacerta vivipara	Wild	2.94	11			0.0006		Voituron et al., 2006
Reptiles	Trachemys scripta	Captivity	240	41.3			0.0130		Willmore and Storey, 1997

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Table 3. Descriptive statistics of biochemical metrics of oxidative status included in this study. Values of Greenland shark (GS) are also reported separately for comparison with the whole variation of our sample. N = number of vertebrate species data were collected from; SD = standard deviation; rbcs = red blood cells; PCs = protein carbonyls (expressed as nmol/mg proteins); GPX = glutathione peroxidase (expressed as Units/mg proteins); res = residuals; NoBM = no correction for body mass; BMcorr = correction for body mass.

	PCs muscle	PCs rbcs	GPX muscle	GPX rbcs	resPC muscle NoBM	resPC muscle BMcorr	resPCrbcs NoBM	resPCrbcs BMcorr	resGPXmuscle NoBM	resGPXmuscle BMcorr	resGPXrbcs NoBM	resGPXrbcs BMcorr
N	16	8	15	10	16	16	8	8	15	15	10	10
Mean	1.8	5.7	0.032	0.312	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
SD	1.9	3.4	0.056	0.267	0.058	0.054	0.121	0.107	0.009	0.008	0.054	0.044
Min	0.2	1.1	0.001	0.007	-0.149	-0.118	-0.242	-0.221	-0.010	-0.010	-0.102	-0.056
Max	6.3	11.0	0.210	0.724	0.141	0.152	0.189	0.131	0.019	0.021	0.083	0.081
Perc. 25	0.4	3.3	0.004	0.037	-0.019	-0.018	-0.040	-0.039	-0.008	-0.006	-0.040	-0.039
Perc. 50	0.9	4.7	0.011	0.286	-0.001	0.000	0.009	0.018	0.000	0.000	0.000	0.000
Perc. 75	3.3	9.1	0.025	0.588	0.027	0.014	0.050	0.078	0.003	0.004	0.047	0.035
GS	*5.5*	*4.7*	*0.094*	*0.007*	*0.031*	*0.007*	*-0.009*	*-0.040*	*0.015*	*0.010*	*0.000*	*0.000*

Figure captions

Figure 1. Scatterplots of the residuals of maximum lifespan (corrected for body mass in all panels) against those of metrics of oxidative status (see text). None of the biochemical metrics of oxidative status were significantly correlated to maximum lifespan, regardless of whether body mass was or was not included in the models. The Greenland shark is shown as a blue dot in each graph.

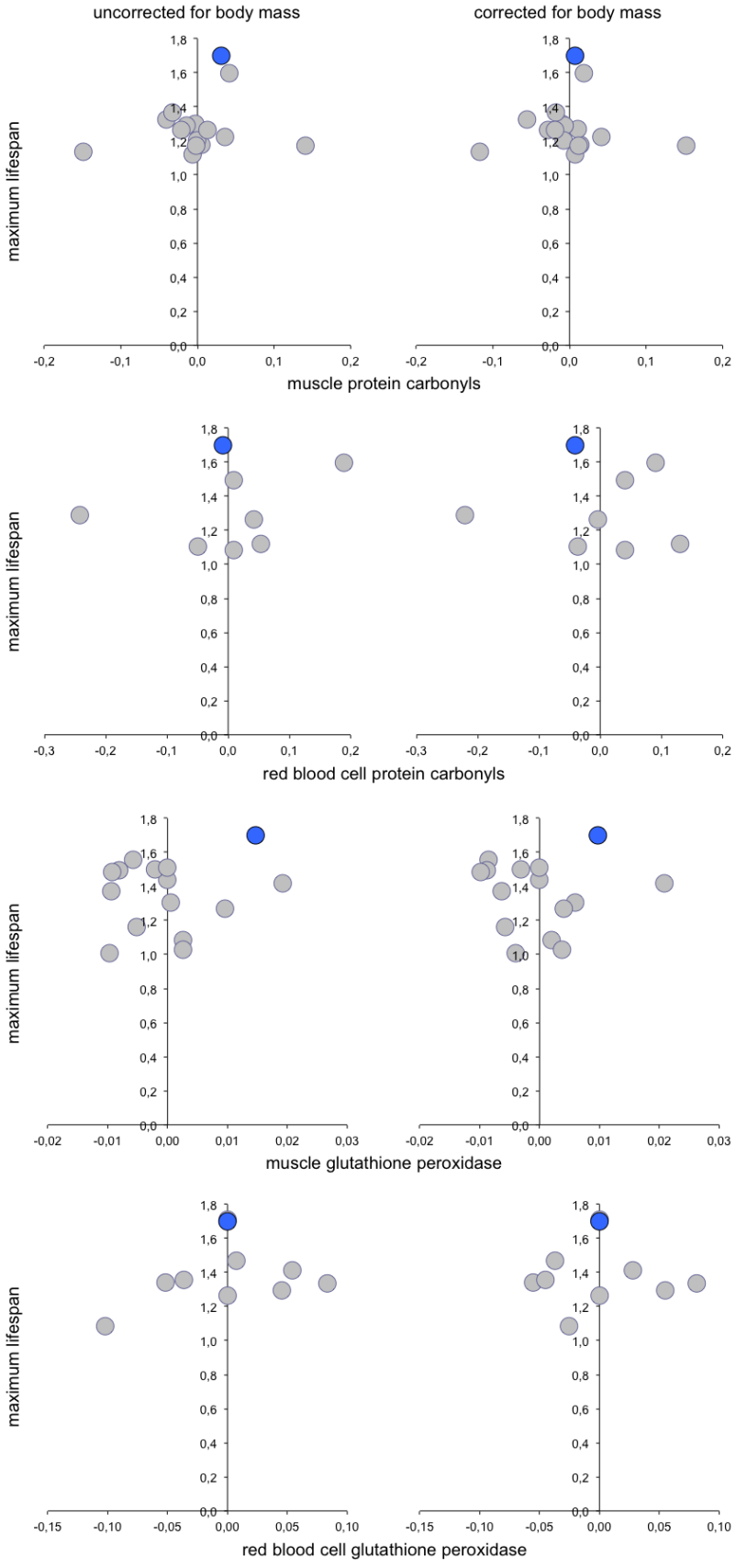
uncorrected for body mass
corrected for body mass
maximum lifespan
muscle protein carbonyls
red blood cell protein carbonyls
muscle glutathione peroxidase
red blood cell glutathione peroxidase

Figure 1